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CO2 exchange characteristics during dark-light transitions in wild-type and mutant Chlamydomonas reinhardii cells

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Abstract
A burst of net CO2 uptake was observed during the first 3–4 min after the onset of illumination in both wild-type *Chlamydomonas reinhardii* in which carbonic anhydrase was chemically inhibited with ethoxyzolamide and in a mutant of *C. reinhardii* (*ca*-1-12-1C) deficient in carbonic anhydrase activity. The burst was followed by a rapid decrease in the CO2 uptake rate so that net evolution often occurred. After a 2–3 min period of CO2 evolution, net CO2 uptake again increased and ultimately reached a steady-state, positive rate. From [14CO2]-tracer studies it was determined that CO2 fixation proceeded at a nearly linear rate throughout the period of illumination. Thus, prior to reaching a steady state, there was a rapid accumulation of inorganic carbon inside the cells which apparently reached a supercritical concentration and the excess was excreted, causing a subsequent efflux of CO2. A post illumination burst of net CO2 efflux was also observed in ethoxyzolamide-inhibited wild type and *ca*-1 mutant cells, but not in the unihibited wild type. [14CO2]-tracer experiments revealed that this burst was the result of a collapse of a large internal inorganic carbon pool at the onset of darkness rather than a photorespiratory post-illumination burst. These results indicate that upon illumination, chemical or genetic inhibition of carbonic anhydrase initially causes an accumulation of excess inroganic carbon in *C. reinhardii* cells, and that unknown regulatory mechanisms correct for this imbalance by first excreting the excess inorganic carbon and then, after several dampened oscillations, achieving an equilibrium between bicarbonate uptake, bicarbonate dehydration, and CO2 fixation.

Keywords
bicarbonate transport, Chlamydomonas, CO2 Exchange, mutants, photosynthesis

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CO_2 exchange characteristics during dark-light transitions in wild-type and mutant *Chlamydomonas reinhardii* cells

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Abstract. A burst of net CO_2 uptake was observed during the first 3–4 min after the onset of illumination in both wild-type *Chlamydomonas reinhardii* in which carbonic anhydrase was chemically inhibited with ethoxyzolamide and in a mutant of *C. reinhardii* (*ca*-1-12-1C) deficient in carbonic anhydrase activity. The burst was followed by a rapid decrease in the CO_2 uptake rate so that net evolution often occurred. After a 2–3 min period of CO_2 evolution, net CO_2 uptake again increased and ultimately reached a steady-state, positive rate. From [$^{14}CO_2$]-tracer studies it was determined that CO_2 fixation proceeded at a nearly linear rate throughout the period of illumination. Thus, prior to reaching a steady state, there was a rapid accumulation of inorganic carbon inside the cells which apparently reached a supercritical concentration and the excess was excreted, causing a subsequent efflux of CO_2. A post illumination burst of net CO_2 efflux was also observed in ethoxyzolamide-inhibited wild type and *ca*-1 mutant cells, but not in the unihibited wild type. [$^{14}CO_2$]-tracer experiments revealed that this burst was the result of a collapse of a large internal inorganic carbon pool at the onset of darkness rather than a photorespiratory post-illumination burst. These results indicate that upon illumination, chemical or genetic inhibition of carbonic anhydrase initially causes an accumulation of excess inroganic carbon in *C. reinhardii* cells, and that unknown regulatory mechanisms correct for this imbalance by first excreting the excess inorganic carbon and then, after several dampened oscillations, achieving an equilibrium between bicarbonate uptake, bicarbonate dehydration, and CO_2 fixation.

Introduction

Unicellular green algae, including *Chlamydomonas reinhardii*, and cyanobacteria possess CO_2-concentrating systems which saturate their photosynthetic capacity at otherwise limiting external CO_2 concentrations [1–4, 13]. The mechanism responsible for this effect requires energy [1], is light dependent [7], results in an accumulation of intracellular inorganic carbon to a concentration several-fold higher than that external to the cells [1, 2, 4, 13], involves a saturable transport system for inorganic carbon [8], probably involves the electrogenic transport of bicarbonate [5], and includes carbonic anhydrase (EC 4.2.1.1) as an essential component of the system [9].

Two *C. reinhardii* mutants deficient in some portion of the CO_2-concentrating system have been described [9, 10]. Based principally on the physiological analysis of a mutant deficient in carbonic-anhydrase activity (strain *ca*-1-12-1C), the major role of carbonic anhydrase in the CO_2-concentrating system was concluded to be dehydration of transported bicarbonate to supply CO_2 for photosynthesis [9]. A second mutation affecting this system (strain *pmp*-1-16-5K) has been characterized as resulting in a deficiency in bicarbonate transport [10]. A double mutant (strain *ca pmp*) has also been constructed genetically and characterized physiologically [11].

In this paper we describe a transient accumulation of inorganic carbon observed following a dark to light transition in *C. reinhardii* cells in which internal carbonic anhydrase activity is either inhibited chemically or deficient genetically.

Materials and methods

Chlamydomonas reinhardii wild-type strain 2137 *mt* + and mutant strains *ca*-1-12-1C, *pmp*-1-16-5K, and the double mutant *ca pmp* were grown in minimal medium in liquid culture [9]. Wild-type cells were grown without CO_2 enrichment, but mutant cells were aerated with 5% CO_2 in air. The CO_2 enrichment was discontinued two days prior to analysis in order to allow induction of the CO_2-concentrating system [7].

Net CO_2 exchange was monitored by infrared gas analysis in an open system [9]. A similar but closed system was utilized for $^{14}CO_2$ incorporation studies. The closed system included two 20 l glass bottles to minimize CO_2 concentration changes during the course of experiments. Two mCi of $NaH^{14}CO_3$ were injected into H_3PO_4 in a 25-ml, in-line vessel. Air (367 $\mu l\,l^{-1}$ CO_2) was pumped through the closed system at approx. 350 ml min^{-1} by a peristaltic pump for 4 h in the absence of algae to ensure complete mixing and equilibration of the $^{14}CO_2$. A concentrated suspension of algae was introduced into a pre-equilibrated buffer solution (MOPS-KOH, pH7.0, 25C) in the dark 5 min prior to illumination (500 $\mu Em^{-2}\,s^{-1}$). The total volume of the algal suspension was initially 30 ml (10 μg Chl ml^{-1}), and duplicate 200 μl samples were removed by syringe at timed intervals and subjected to silicone oil filtering centrifugation to determine fixed ^{14}C and internal accumulation of [^{14}C] inorganic carbon [8]. Accumulation data were converted from $\mu g(mg\,Chl)^{-1}$ to mM internal concentrations as previously described [1, 8]. Monitoring of inorganic ^{14}C in the medium during the course of experiments indicated no significant variation in concentration. Final CO_2 concentrations were 366 $\mu l\,l^{-1}$ for wild type plus ethoxyzolamide (EZA) and 352 $\mu l\,l^{-1}$ for wild type.

Chlorophyll concentration was determined after extraction into 96% ethanol [12].

Results and discussion

The CO_2-exchange characteristics of wild-type *C. reinhardii* in the presence or absence of the carbonic anhdyrase inhibitor ethoxyzolamide (EZA) are illustrated in Fig. 1A. In the absence of the inhibitor, the cells exhibited a nearly square-form change from CO_2 efflux to CO_2 uptake on the transition from dark to light and the reverse upon the transition from light to dark. In the presence of EZA a burst of CO_2 uptake was observed during the first 3–4 min after the dark to light transition and was followed by a reduced rate of uptake which often was so extreme that net CO_2 efflux occurred.

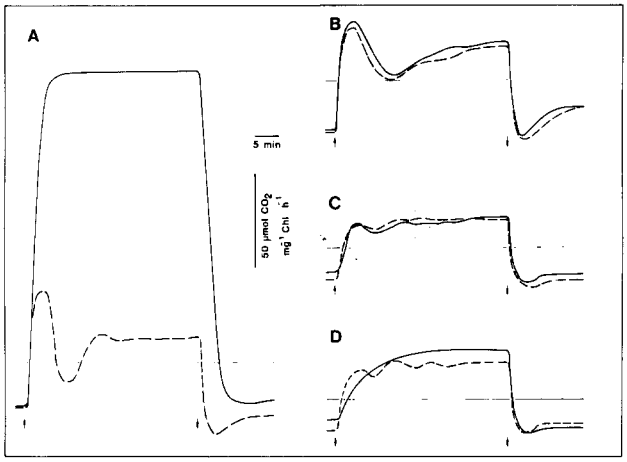

Figure 1. Net CO_2-exchange patterns of *Chlamydomonas reinhardii* A) wild type (2137 *mt* +), B) carbonic anhydrase deficient mutant (*ca*-1-12-1C), C) double mutant (*ca pmp*) and D) bicarbonate transport deficient mutant (*pmp*-1-16-5K). Patterns were recorded either with (dashed line) or without (solid line) the addition of 50 μM ethoxyzolamide in the dark 5 min prior to illumination. Net CO_2 exchange was monitored at 25 °C, with 350 μl CO_2 and 21% O_2. Arrows indicate light on (↑) and light off (↓) and zero CO_2 exchange is indicated by the fine horizontal line.

Following this efflux the net rate of CO_2 uptake rose, occasionally with minor oscillations, to a steady state level roughly 10-fold lower than the uninhibited rate. Following the light to dark transition, a marked post-illumination burst of CO_2 efflux was observed in the presence of EZA before a steady-state rate of dark CO_2 efflux was attained.

The CO_2-exchange characteristics of the carbonic anhydrase-deficient mutant *ca*-1-12-1C in both the presence and absence of EZA were similar to those of wild type inhibited with EZA (Fig. 1B). These CO_2-exchange characteristics were unaffected by the addition of an excess of bovine erythrocyte carbonic anhydrase (data not shown), demonstrating that the observed pattern of CO_2-exchange was not due to disequilibration between CO_2 in the gas and aqueous phases.

Fig. 1D illustrates the CO_2-exchange characteristics of the transport deficient mutant. Oscillations were apparent with the EZA-inhibited cells, but they were not as dramatic as with inhibited wild-type cells (Fig. 1A). Inhibition of the steady-state photosynthetic rate by EZA was also less pronounced with the transport-deficient mutant than with wild type, but the rate of photosynthesis in the presence of EZA was similar for both wild-type and mutant cells. The post-illumination CO_2 efflux was increased slightly in the EZA-inhibited cells of the *pmp*-1 mutant.

As with the *ca*-1 mutant, the CO_2-exchange characteristics of the double mutant (*ca pmp*) were essentially unaffected by EZA (Fig. 1C). The CO_2-exchange pattern of the double mutant was very similar to that of the EZA-inhibited *pmp*-1 mutant (Fig. 1D). Since the *ca*-1 mutation apparently results in a deficiency of internal carbonic anhydrase activity [9], the combination of this mutation with the *pmp*-1 mutation in the double mutant would be expected to behave similarly to the *pmp*-1 mutant with carbonic anhydrase inhibited [11].

In order to better understand the nature of the burst of CO_2 uptake observed upon illumination in the *ca*-1 mutant and EZA-inhibited wild type, and to a lesser extent in the double mutant and EZA-inhibited *pmp*-1 mutant (Fig. 1), $^{14}CO_2$ uptake into both inorganic and fixed carbon was monitored under conditions similar to those of the CO_2-exchange measurements. In unhibited wild-type cells both total ^{14}C uptake and incorporation of ^{14}C into fixed carbon increased linearly with time upon illumination (Fig. 2A) as might be expected from the CO_2-exchange pattern (Fig. 1A). Internal inorganic carbon increased from less than 0.5 mM in the dark to 2–4 mM in the light, then dropped immediately back to the dark level when the light was terminated (Fig. 2A).

In the presence of EZA, however, the total uptake of $^{14}CO_2$ into the wild-type cells exhibited a rapid rate of uptake upon illumination (Fig. 2B), very similar to that observed with CO_2-exchange. This burst of CO_2 uptake was apparently due entirely to uptake into an internal inorganic carbon pool, as incorporation of ^{14}C into fixed carbon proceeded at a much lower, nearly linear rate in the light. The internal inorganic carbon concentration increased to about 25 mM during the first 3–4 min of illumination, but the concentration subsequently dropped to a steady-state level of approximately 10 mM. This steady state level of internal inorganic carbon is similar to the level observed previously in short-term experiments [9]. It is apparent that inorganic carbon was actually being released from the cells during the drop in internal concentration from 4–7 min after illumination. The total ^{14}C content of the cells actually decreased even though fixation of ^{14}C continued at a relatively steady rate. This release of inorganic carbon from the cells corresponds to the net CO_2 efflux observed in the CO_2-exchange measurements at about 5 min after illumination (Fig. 1A).

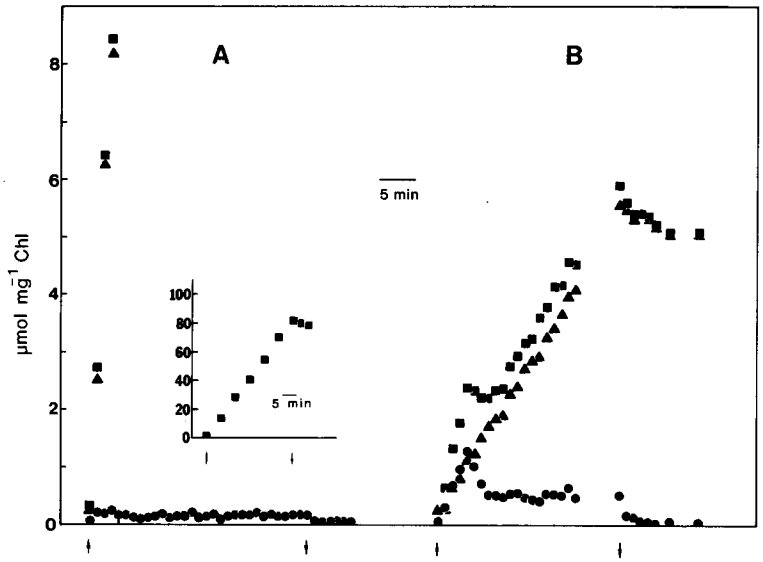

Figure 2. Uptake of $^{14}CO_2$ into inorganic (●), fixed (▲), and total (■) carbon pools in wild type *Chlamydomonas reinhardii* either in the absence (A) or in the presence (B) of 50 µM ethoxyzolamide. Other conditions were as indicated for Fig. 1. Arrows indicate light on (↑) and light off (↓). Inset in (A) indicates time course, on an expanded scale, of total carbon uptake (■) in the absence of ethoxyzolamide.

After the light to dark transition, the high internal inorganic carbon pool of the EZA-inhibited wild-type cells returned to the dark level (less than 0.5 mM) within about 3 min (Fig. 2B). The amount of fixed ^{14}C also decreased when illumination was discontinued, but this was entirely accounted for by excretion of fixed carbon into the medium (data not shown). These observations indicate that the post-illumination burst of CO_2 efflux observed in the CO_2-exchange experiments (Fig. 1A) was due primarily to release of the internal inorganic carbon pool rather than release of newly-fixed carbon (i.e., photorespiration). The characteristics of $^{14}CO_2$ incorporation and distribution in the *ca*-1 mutant were very similar to those of the EZA-inhibited wild type (data not shown).

It is apparent that a deficiency in carbonic anhydrase leads to excessive accumulation of internal inorganic carbon upon illumination, and that the resultant state of disequilibrium is relieved by excretion of this excess bicarbonate. The mechanism responsible for these observations is unknown. In the presence of carbonic anhydrase, HCO_3^- accumulates and OH^- is not released. Since OH^- is thought to be the anion excreted in a loosely coupled exchange for HCO_3^- [6], lack of HCO_3^- dehydration would cause a charge imbalance between the inside and outside of the cell, perhaps leading to an extreme membrane hyperpolarization which could drive the efflux of HCO_3^-. Although HCO_3^- uptake normally causes some membrane hyperpolarization [5], the extreme hyperpolarization suggested here has not been observed.

Apparently less HCO_3^- accumulated in the EZA-inhibited *pmp*-1 mutant and the double mutant, as suggested by a much smaller burst of net CO_2 uptake upon illumination than observed in EZA-inhibited wild type and the *ca*-1 mutant (Fig. 1). This conclusion is consistent with the *pmp*-1 mutation causing a deficiency in the HCO_3^- uptake mechanism [10], since a reduced HCO_3^- transport rate might give the cell time to react before the HCO_3^- overshoot became as extreme as in the presence of a fully functional HCO_3^- transporter.

Following the initial rapid uptake and excretion of bicarbonate, the EZA-inhibited, wild-type cells apparently pass through a series of increasingly dampened oscillations between HCO_3^- uptake and excretion (Fig. 1). These oscillations probably represent progress by the cell in achieving equilibrium between the rates of HCO_3^- uptake, HCO_3^- dehydration, and CO_2 fixation, but again the mechanistic details regulating the response are presently unknown.

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